Nanoparticle exposure activates an inositol trisphosphate receptor-dependent elevation of reactive oxygen species and apoptosis in human alveolar lung cells

Sarah Alvarado, Brianna Manes, Dr. Jay Brewster
Summer Undergraduate Research in Biology, 2013
Pepperdine University, Malibu, California 90263

Summer Undergraduate Research in Biology
Pepperdine University

PEPPERDINE UNIVERSITY

NSF

Abstract

The signaling of cell stress in response to organelle dysfunction, toxin exposure, and mutation is complex; generating responses that can include adaptation, or in sever cases, cellular apoptosis. Nanoparticles (20-100 nm diameter) have been shown to induce cell stress in lung cells, potentially identifying a cause of lung disease in areas with high levels of particulate-based air pollution. This study examines the effect of carbon black (CB) and titanium dioxide (TiO_2) nanoparticles on stress signaling and apoptosis in cultured A549 human alveolar epithelial cells. CB and TiO_2 powders were dispersed throughout a buffered solution containing bovine serum albumin using probe sonication. Particle size analysis was performed, revealing stable nanoparticle complexes ranging from 75 nm for CB and from 172 nm for TiO_2. A range of nanoparticle doses between 5 and 100 µg/mL were evaluated for toxicity using a visual inspection for DAPI-stained apoptotic nuclei. This assay revealed a peak of cell death activation at 75 µg/mL for CB and 100 µg/mL for TiO_2 though the CB was more effective at inducing apoptosis than TiO_2. A live/dead-cell fluorescent protease assay confirmed CB to significantly decrease cell viability. Further studies revealed acute CB exposure, but not TiO_2 exposure, to induce reactive oxygen species (ROS). Interestingly, inhibition of nanoparticle-induced calcium release by the inositol triphosphate receptor (ITPR) inhibited ROS production, suggesting a role for ER Ca^{2+} stores in activating ROS production. Further research is underway to determine which stress/apoptotic signaling pathways are induced downstream of nanoparticle exposure.

Introduction

The endoplasmic reticulum (ER) is an organelle within eukaryotic cells that extends outward from the nucleus through the cytoplasm. It is the site of protein folding and modification, including glycosylation and formation of disulfide bonds, as well as the site of storage of free calcium, Ca^{2+}. In the event of altered homeostasis of the conditions in the ER lumen, proteins may stop folding correctly, which leads to the accumulation of unfolded proteins, and activates the unfolded protein response (UPR) [10].

Recent studies have demonstrated a link between cigarette smoke and particulate matter (PM) pollution in the air to the increase in cardiovascular linked death and diseases by impacting ER stress signaling pathways within cells. Cigarette smoke consists of a mixture of gases and particulate matter including reactive oxygen species (ROS). Pollution particulate matter may consist of metals, salts, carbonaceous material, and is primarily caused by traffic-related combustion. The molecular pathways through which both cigarette smoke and PM cause cytotoxicity and illnesses are not well understood [4,5].

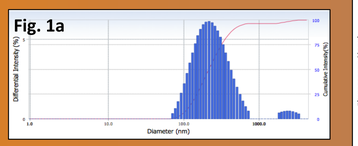
It has been shown that cells, especially human lung epithelial cell, absorb small particulate matter, and ultra fine particles that are cytotoxic to cells. However, the endocytic mechanism is still largely unknown [16]. Carbon black (CB) is mostly pure, ultra-fine carbon material found in the air due to pollution by combustion and industry, and it is known to be one of the main components of atmospheric pollution particles. Titanium dioxide (TiO_2) is a naturally occurring mineral, but industries use an ultra fine powder form as a pigment in many cosmetic products such as sunscreen and toothpaste.

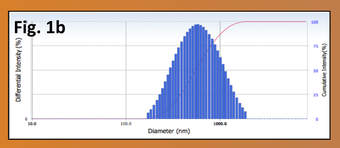
While our cells are exposed to cytotoxic ultra fine particulates on a daily basis, our cells have developed an advanced defense mechanism to ameliorate the stress induced by common toxic particles, CB and TiO_2. NRF2, a transcription factor in the cytosol, has been shown to be activated as a result of a redox imbalance, which can be characterized as an accumulation of ROS in the cytosol, ER and mitochondria [9]. As a result of the accumulation of free radicals, NRF2 is activated by ROS directly and proceeds to translocate to the nucleus where it binds to the ARE, antioxidant response element, and upregulate enzymes for scavenging ROS and general synthesis of essential glutathione: HO-1, NADPH and GCLc [2]. It is expected that the enzymes and antioxidants would be upregulated under particulate induced stress, however, there has not been consistent evidence to support this theory. If HO-1, NADPH ad GCLc cannot mitigate the cell's redox dysfunction, the apoptotic intrinsic pathway through the mitochondria is activated resulting in cell death[11].

The aim of this study is to characterize the affects of CB and TiO_2 on A549 human alveolar lung cells and the particle's ability to induce oxidative stress, caspase activation and apoptosis. Furthermore, we will be evaluating the cell's defense response to the toxic particles by investigating Nrf2 activation as well as the antioxidants and enzymes downstream of the vital redox transcription factor.

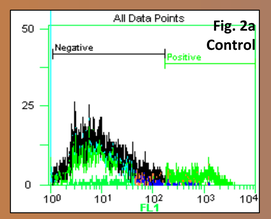
Results

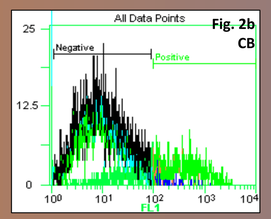
Fig. 1 Probe sonication techniques distribute carbon black and titanium dioxide in solution on the nanoparticle scale as tested by Micromeritics Analytical Services (Norcross, GA). A mixture of particles (CB or TiO_2) at 1 mg/mL, bovine serum albumin (BSA) in PBS at 6.5 mg/mL, and distilled water was sonicated for 5 minutes at 20% amplitude of a 500 W ultrasonic probe. CB particles exhibited the largest range of diameters from 76.2 nm to 755.6 nm. (a) CB (b)TiO_2





Results





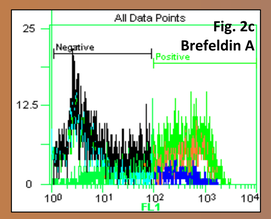
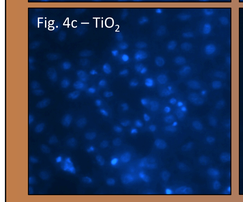
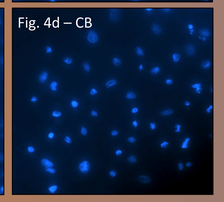
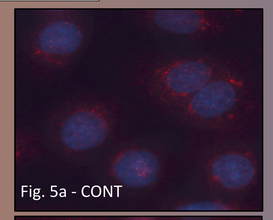
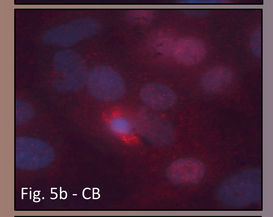


Fig. 4 Carbon black, titanium dioxide, and ER stressor Brefeldin A cause cell death. As described in Figure 3, cells were seeded and poisoned, fixed and imaged, and the most death seen is with Brefeldin A after 36 hours. Both titanium dioxide and carbon black cause significant levels of cell death as well. Condensed, brightly fluorescing nuclei are considered apoptotic while flat, wide nuclei are alive.
(A) Control, (B) Brefeldin A, (C) TiO_2, (D) CB.









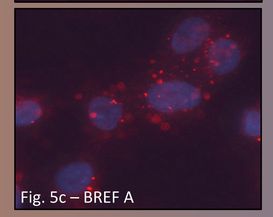
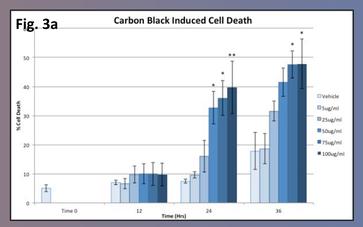


Fig. 5 Executioner caspase 3 is activated due to CB exposure for 36 hours. A549 human alveolar epithelial cells were grown on poly-lysine treated coverslips, poisoned for 36 hours, stained with an anti-active caspase 3 antibody, mounted with Antifade Gold with DAPI mounting media, and imaged on a Nikon Eclipse TI microscope. (a) A549 cells treated with vehicles of sonicated protein and DMSO. (b) A549 cells treated with 100 µg/mL CB. (c) A549 cells treated with 1 µM Brefeldin A, an ER stressor that inhibits protein translocation from the ER to the golgi, resulting in ER stress and activation of the UPR and stress-induced apoptosis.

Fig. 10 Carbon black shows a trend toward increasing cytotoxicity and significantly decreases cell viability. A549 cells were treated with CB for 36 hours and analyzed by a commercial kit that measured protease activities. Cytotoxicity and cell viability were measured by cleavage of a cell-impermeant peptide substrate by a dead cell protease, and cleavage of cell-permeant peptide substrate by a live cell protease, respectively. (a) Cytotoxicity shows an increasing trend due to CB. (b) CB decreases cell viability. . Values represent ±SE, ANOVA, Tukey's Post-hoc analysis *p<0.05, **p<0.01, ***p<0.001.

Results

Fig. 2 Carbon black and Brefeldin A affect apoptosis levels as indicated by a fluorescent Annexin V apoptotic marker through flow cytometry. A549 cells were poisoned with CB, Brefeldin A (apoptotic inducer), or DMSO/ sonicated BSA control for 24 hours, stained with fluorescent Annexin V, and run on a flow cytometer. Annexin V "positive" cells are considered apoptotic. CB and Brefeldin A affect levels of apoptosis as compared to control. Replicates are in progress. (A) Control, (B) CB, and (C) Brefeldin A.



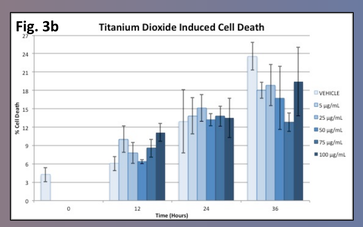


Fig. 2 Carbon black and titanium dioxide particulates cause cell death. A549 cells were seeded into a 24-well plate and poisoned for 0, 12, 24, and 36 hours. The select cells were fixed in four percent paraformaldehyde, stained with DAPI nuclear fluorescent stain, and imaged on a Nikon Eclipse TI microscope. At least two images were taken and at least 500 cells were counted per well, noting nuclear morphology for each cell. (a) Carbon black was probe sonicated and treated to cells at multiple concentrations. The highest percentage of cell death with carbon black occurred with 100 µg/mL after 36 hours. (b) Titanium dioxide was probe sonicated and treated to cells at multiple concentrations; however, no trend toward cell death by titanium dioxide was observed. Values represent ±SE, ANOVA *p<0.05, **p<0.01, CB: n=4, TiO_2: n=3, significance shown is treated compared to control at each time .

Fig. 6 Cell protection through antioxidant defense mechanisms induced by oxidative stress. Reactive oxygen species directly interacts with NRF2 and causes the dissociation of NRF2 from KEAP1 and translocation to the nucleus, where its binds the ARE binding domain resulting in upregulation of enzymes and antioxidants responsible for returning the cell to redox homeostasis.

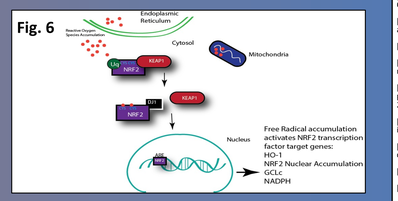
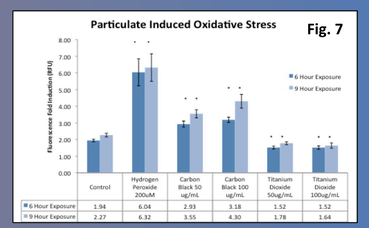
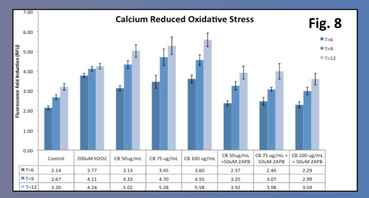


Fig. 7 Carbon black induces significant levels of reactive oxygen species. A549 cells were treated with sonicated carbon black and titanium dioxide, as well as hydrogen peroxide, for 6 and 9 hours. The cells were loaded with carboxylated DCFH-DA dye and scanned with a fluorescent plate reader. Titanium dioxide did not demonstrate any levels of ROS. Values represent ±SE, Wilcoxon Mann-Whitney, p≤0.05, n=6.



	Control	Hydrogen Peroxide 200uM	Carbon Black 50 ug/mL	Carbon Black 100 ug/mL	Titanium Dioxide 50ug/mL	Titanium Dioxide 100ug/mL
6 Hour Exposure	1.94	6.04	2.93	3.18	1.52	1.52
9 Hour Exposure	2.27	6.32	3.55	4.30	1.78	1.64

Fig. 8 Calcium release blocker 2-APB decreases CB induced ROS accumulation. A549 cells treated with varying concentrations of CB had lower amounts of ROS with 2-APB than without.



	Control	200uM H2O2	CB 50ug/mL	CB 75 ug/mL	CB 100 ug/mL	CB 50ug/mL +50uM 2APB	CB 75 ug/mL + 50uM 2APB	CB 100 ug/mL + 50uM 2APB
T=6	2.14	3.77	3.13	3.45	3.60	2.37	2.46	2.29
T=9	2.67	4.11	4.33	4.70	4.55	3.25	3.07	2.99
T=12	3.20	4.24	5.02	5.28	5.58	3.92	3.98	3.59

Fig. 9

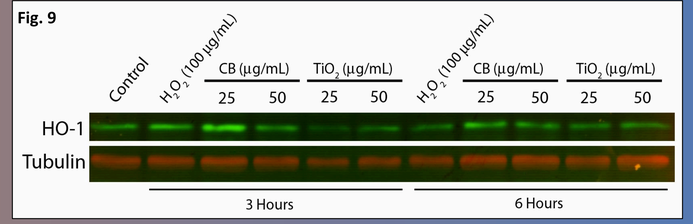


Fig. 9 Carbon black causes upregulation of HO-1. A549 cells were treated with sonicated CB and TiO_2, as well as hydrogen peroxide, for 3 and 6 hours. Plates were scraped with lysis buffer, triturated, and run on a western blot. The blots were then washed in primary antibodies conjugated to HO-1 and tubulin, then fluorescent secondary antibodies, and scanned on a LICOR infrared scanner. Hydrogen peroxide and CB seemed to cause the highest levels of HO-1, a marker of ARE activation.

Conclusion

- CB and TiO_2 were dispersed in solution resulting in aggregates with size ranges beginning at 76 and 172 nm in diameter, respectively, through probe sonication. The nanoparticles are stable in solution as measured by zeta potential; CB -31.63 and TiO_2 -10.85 mV.
- Carbon black concentrations ranging from 50-100 µg/mL causes significant cell death after 24 and 36 hours whereas equivalent TiO_2 concentrations do not.
- Carbon black treatment ranging from 50-100 µg/mL results in the accumulation of ROS.
- Preliminary results display a trend of 50 µM 2-APB protection against carbon black induced ROS accumulation, indicating the involvement of the ER in particulate induced apoptosis.
- HO-1, a marker of the antioxidant response element, is upregulated after treatment with 25 and 50 µg/mL CB at 3 and 6 hours; however, TiO_2 did not display HO-1 activity.
- Executioner caspase 3 is active after treatment with CB for 36 hours.
- Assessment of other stress signaling pathways is underway.

Works Cited

[1] Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., Walter, P. (2007) Molecular Biology of the Cell. NewYork: Garland Science, Taylor and Francis Group, LLC.

[2] D'Autreaux, B., & Toledano, M. (2007). ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis. Nature 8: 813-822.

[3] Duan, J., Yu, Y., & Li, Y. (2013). Toxic Effect of Silica Nanoparticles on Endothelial Cells through DNA Damage Response via Chk1-Dependent G2/M Checkpoint. PLoS ONE 8: 1-13.

[4] Hussain, S., Thomassen, L.C.J., Ferecatu, I., Borot, M., Andreau, K., Martens, J.A., Fleury, J., Baeza-Squiban, A., Marano, F., Boland, S. (2010) Carbon black and titanium dioxide nanoparticles elicit distinct apoptotic pathways in bronchial epithelial cells. Particle and Fibre Toxicology 7:10.

[5] Jia, X., Hao, Y., Guo, X. (2012) Ultrafine carbon black disturbs heart rate variability in mice. Toxicology Letters 211: 274-280.

[6]Jorgensen, E., Stinson, A., Shan, L., Yang, J., Gietl, D., Albino, A.P. (2008) Cigarette smoke induces endoplasmic reticulum stress and the unfolded protein response in normal and malignant human lung cells. BMC Cancer 8: 229.

[7] Laing, S., Wang, G., Briazova, T., Zhang, C., Wang, A., Zheng, Z., Gow, A., Chen, A.F., Rajagopalan, S., Chen,L.C., Sun, Q., Zhang, K. (2010) Airborne particulate matter selectively activates endoplasmic reticulum stress response in the lung and liver tissues. American Journal of Physiology – Cell Physiology 299: C736-C749.

[8] Malhotra, J.D., Miao, H., Zhang, K., Wolfson, A., Pennathur, S., Pipe, S.W., Kaufman, R.J. (2008) Antioxidants reduce endoplasmic reticulum stress and improve protein secretion. Proceedings of the National Academy of Sciences of the United States of America 105(47): 18525-18530.

[9] Messier, E., Bahmed, K., Tuder, R., & Kosmider, B. (2013). Trolox contributes to NRF2-mediated protection of human and murine primary alveolar type II cells from injury by cigarette smoke. Cell Death and Disease 4: 1-11.

[10] Naidoo, Nirinjini. (2009) ER and aging – Protein folding and the ER stress response (Review). Ageing Research Reviews 8: 150–159.

[11] Niture, S., Khatri, R., & Kaiswal, A. (2013). Regulation of Nrf2-an update. Free Radical Biology and Medicine 2: 2-6.

[12] Rutkowski, D.T., Kaufman, R.J. (2007) That which does not kill me makes me stronger: adapting to chronic ER stress (Review). TRENDS in Biochemical Sciences 32(10): 469-476.

[13] Scott, Jessica. (2008) IRE1 signaling between the endoplasmic reticulum and the nucleus. Pepperdine University Senior Honors Thesis.

[14] Sun, Q., Tan, D., Zhou, Q., Liu, X., Cheng, Z., Liu, G., Sang, X., Gui, S., Cheng, J., Hu, R., Tang, M., Hong, F. (2012) Oxidative Damage of lung and it's protective mechanism in mice caused by long-term exposure to titanium dioxide nanoparticles. J Biomed Mater Res A.

[15] Trinh, Van T. (2008) Inositol 1,4,5-Triphosphate (IP3) – Receptor Mediated Signaling Following Endoplasmic Reticulum Stress. Pepperdine University Senior Honors Thesis.

[16] Watterson, T.L., Hamilton, B., Martin, R., Coulombe, R.A. Jr. (2009) Urban Particulate Matter Causes ER Stress and the Unfolded Protein Response in Human Lung Cells. Toxicological Sciences 112(1): 111-122.

[17] Xiao, Y., Forry, S.P., Gao, X., Holbrook, R.D. Telford, W.G., Tona, A. (2010) Dynamics and mechanisms of quantum dot nanoparticle cellular uptake. Journal of Nanobiotechnology 8:13.

[18] Ze, Y., Zheng, L., & Zhao, X. (2013). Molecular Mechanism of titanium dioxide nanoparticles-induced oxidative injury in the brain of mice. Chemosphere 9: 1183-1189.

Acknowledgments

This research was funded by the National Science Foundation, Research Experience for Undergraduates, REU-Site Grant, #DBI-1062721, an Undergraduate Research Fellowship, the Pepperdine University Summer Undergraduate Research Program, and the Natural Science Division of Pepperdine University. We would like to thank our lab mates Vicki Mercado, Agustin Vargas, and Thomas Addington, as well as our mentor Dr. Brewster for their guidance, advice, and help in our project this summer. We would also like to thank the Pepperdine University Natural Science Division for their work and dedication to this program.